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# Expression and Purification of a Cathelicidin-Derived Antimicrobial Peptide, CRAMP

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**Abstract** Application of recombinant protein production and particularly their isotopic enrichment has stimulated development of a range of novel multidimensional heteronuclear NMR techniques. Peptides in most cases are amenable to assignment and structure determination without the need for isotopic labeling. However, there are many cases where the availability of $^{15}N$ and/or $^{13}C$ labeled peptides is useful to study the structure of peptides with more than 30 residues and the interaction between peptides and membrane. CRAMP (Cathelicidin-Related AntiMicrobial Peptide) was identified from a cDNA clone derived from mouse femoral marrow cells as a member of cathelicidin-derived antimicrobial peptides. CRAMP was successfully expressed as a GST-fused form in *E. coli* and purified using affinity chromatography and reverse-phase chromatography. The yield of the CRAMP was 1.5 mg/1 l. According to CD spectra, CRAMP adopted α-helical conformation in membrane-mimetic environments. Isotope labeling of CRAMP is expected to make it possible to study the structure and dynamic properties of CRAMP in various membrane systems.

**Key words:** CRAMP, antimicrobial peptide, expression, purification, NMR

Antimicrobial peptides constitute a major component of the innate immunity and defense of diverse hosts as plants, invertebrates, and vertebrates including human [3]. Cathelicidins are a major class of antimicrobial peptides characterized by a conserved anionic N-terminal prepro sequence termed cathelin. Conservation of the cathelin sequence indicates that various members of the family evolved from the duplication and modification of a common ancestor gene. CRAMP (Cathelicidin-Related AntiMicrobial Peptide) contains 34 amino acids (GLLRKGGEKIGEKL-KKIGQKIKNFFQKLVPQPEQ), and has potent antimicrobial activity against Gram-positive and Gram-negative bacteria, but no hemolytic activity against human erythrocytes. CRAMP at 1 mM directly causes an immediate permeabilization of the inner membrane of *Escherichia coli*. Antiserum against CRAMP revealed abundant expression in myeloid precursors and neutrophils. Similar to cecropin A, 50 mM CRAMP did not display any hemolytic activity against human red blood cell. In addition, CRAMP was found to have potent antibiotic activity against some pathogenic fungi (*Candia alicans* and *Aspergillus fumigatus*) and tumor cells (Jurkat, A549, and K-562) [7, 18].

Because of the need to record recently developed multinuclear multidimensional NMR spectra, a variety of systems have recently been developed to produce proteins uniformly enriched with stable isotopes. However, only a few systems that produce short peptides have been reported. The enrichment of peptides with stable isotopes may be essential to analyze the NMR data of membrane peptides bound to micelles, since most micelle-bound peptides with higher than 3 kDa size adopt an á-helical conformation that gives only poorly dispersed proton resonances. Furthermore, expression of antimicrobial peptide is known to be very difficult, because antimicrobial peptides are toxic to bacteria. Previously, we have determined the structure of CRAMP with a prosequence in TFE, which is known as an α-helix-inducing organic solvent. However, it was impossible to complete resonance assignment of CRAMP in SDS micelle or DPC micelle because of severe resonance overlapping. In order to study the structure and dynamics of CRAMP in various membrane-mimicking environments, gene expression systems for CRAMP were

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developed. In this paper, we describe the method to express and purify the recombinant antimicrobial peptide CRAMP [11, 22].

## MATERIALS AND METHODS

### Construction of the CRAMP Expression Plasmid

The two DNA strands of the CRAMP gene were synthesized by CoreBio(Seoul, Korea): sense primer, 5'-gat ccg gac ttc tcc gca aag gtg ggg aga aga ttg gtg aaa agc tta aga aaa ttg gcc aga aaa tta aga att ttt ttc aga aac ttg tac ctc agc cgg agc agt agc-3; antisense primer, 5'-tcg agc tac tgc tcc ggc tga ggt aca agt ttc tga aaa aaa ttc tta att ttc tgg cca att ttc tta agc ttt tca cca atc ttc tcc cca cct ttg cgg aga agt ccg-3'. The two strands were phosphorylated and annealed, and the resulting double strand was inserted into pGEX4T-3 vector that was linearized by BamHI and XhoI. Because of the BamHI site (GGATCC) in the recombinant plasmid, the CRAMP gene was composed of 36 amino acids including the Gly-Ser sequence at the N-terminus. The ligation mixture was brought into TOP10 competent cells. The desired plasmid was selected and named pGEX4T-3/GST-CRAMP [1, 21, 23].

### Expression of GST-CRAMP Fusion Protein

The pGEX4T-3/GST-CRAMP plasmid was brought into the expression host, Rosetta (DE3) pLysS (Novagen, Madison, WI, U.S.A.). One colony was used as an inoculum to the 100 ml of LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol, and grown overnight in the 37°C shaking incubator. The next morning, the fully grown culture was mixed with 1 l of fresh LB medium supplemented with the same antibiotics. The culture was grown at 30°C, and IPTG was added to 0.5 mM final concentration when $OD_{600}$ was about 1.0. The culture was harvested 4.5 h later, and the cells were resuspended in 20 ml of 10 mM Tris-HCl (pH 8.0) [20].

### Expression of [$^{13}C/^{15}N$]-Labeled CRAMP Fusion Protein

One colony was used as an inoculum to 4 ml of LB medium supplemented with 100 μg/ml ampicillin and 34 μg chloramphenicol, and grown overnight in a 37°C shaking incubator. The fully grown 4-ml culture was used as an inoculum to 100 ml of minimal medium supplemented with the same antibiotics, and the culture was grown overnight. The minimal medium contained $^{15}NH_4Cl$ and/or $^{13}C$-glucose as a sole nitrogen or carbon source. The next morning, this 100-ml culture was mixed with 900 ml of minimal medium. The culture was grown at 30°C, and IPTG was added to 1 mM final concentration when $OD_{600}$ was 1.2. The temperature was switched to 25°C, and the culture was grown for another 11 h and harvested. The cells were resuspended in 20 ml of 10 mM Tris-HCl, pH 8.0.

### Isolation and Purification of Recombinant Proteins

Cells were lysed by freeze-and-thaw, and the DNA was fragmented by ultrasonication. Triton X-100 was added to the final concentration of 1%, and the supernatant after centrifugation at 15,000 rpm and 4°C was retained for 20 min. The supernatant was loaded onto a glutathione-Sepharose 4B column [2 cm×3 cm (PEPTRON), Daejeon, Korea] equilibrated with 10 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl. The column was washed with 10 times the column volume of PBS buffer [3, 19]. After the washing, bound protein was eluted with 20 ml of 10 mM glutathione. CRAMP was cleaved from the GST-CRAMP fusion protein by adding thrombin directly to the eluted fraction [14]. The reaction mixture was incubated for 20 h at 22°C and loaded onto a Resource RPC column (Amersham Biosciences), and the acetonitrile gradient of 0 to 100% was applied to the column by using the AKTA Basic system (Amersham Biosciences). The CRAMP fraction was collected and lyophilized. MALDI-TOF was used to determine the mass of the purified peptide [9, 13, 16].

### Circular Dichroism

CD experiments were performed using a Jasco spectropolarimeter (Tokyo, Japan) using a cell with a 1-mm path length on KBSI. The CD spectra of the 100-μM peptides were recorded at 25°C and 0.1-nm intervals from 190 to 250 nm. To investigate the conformational changes induced by membrane environments, 2,2,2-trifluoroethanol (TFE), and SDS micelles of defined composition were added to the peptides. For each spectrum, the data from four scans were averaged and smoothed CD data were expressed as mean residue ellipticity [θ] given in $deg \cdot cm^2 \cdot dmol^{-1}$ [24].

### NMR Experiments

The NMR sample contained 1 mM $^{15}N$-labeled CRAMP in SDS micelles. The $^1H$-$^{15}N$ heteronulcear single quantum coherence (HSQC) spectrum was collected at 298 K on a Bruker Avance 500 MHz spectrometer at KBSI. The raw data contained 2,048 and 256 complex points in $t_2$ and of $t_1$, respectively. The spectrum was processed with the NMRPipe/nmrDraw software package and analyzed using the Sparky [6, 8].

## RESULTS AND DISCUSSION

### Construction of CRAMP Expression Plasmid

The map of the plasmid pGEX4T-3/GST-CRAMP is shown in Fig. 1. We also tried two other fusion partners, ubiquitin and ketosteroid isomerase (KSI), but could not obtain any good result. The KSI fusion system failed, because of lysis upon induction. In the case of the ubiquitin system, an

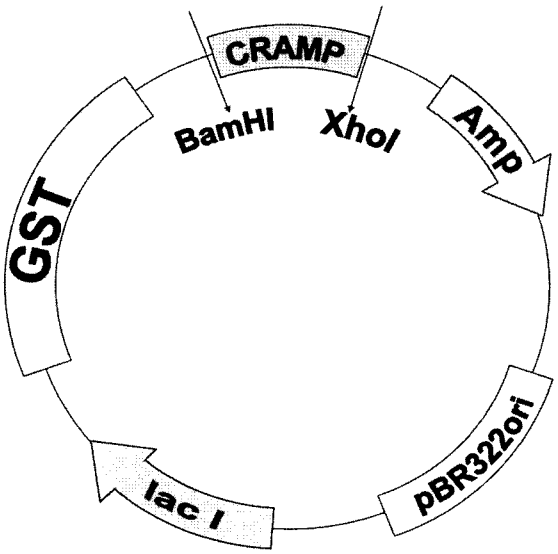


**Fig. 1.** Plasmid map of pGEX4T-3-GST-CRAMP.

unwanted cleavage occurred in the middle of the CRAMP sequence by yeast ubiquitin hydrolase (YUH). Therefore, the GST fusion system appeared to give a result much superior to other fusion systems [10].

### Expression of GST-CRAMP Fusion Protein

As shown in Fig. 2, either the overexpressed or purified GST-CRAMP fusion proteins showed a band corresponding to a molecular mass of 30 kDa, which was consistent with the masses of GST (26 kDa) and CRAMP (4 kDa). To determine optimal conditions, we tried several temperatures for cell growth and various $OD_{600}$ values for induction. The optimal temperature and $OD_{600}$ were determined to be 30°C and 1.0, respectively. We further optimized the post-induction growth condition by lowering the temperature to 25°C [2, 5].

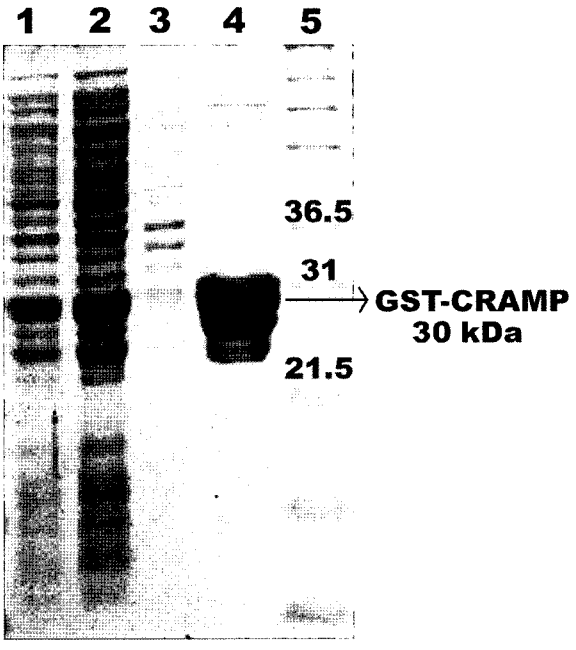


**Fig. 2.** 14% SDS-PAGE along the purification steps of CRAMP. Lane 1, lysate extracted from *E. coli*; lane 2, supernatant of the crude lysate extracted from *E. coli*; lane 3, pellet of the crude lysate extracted from *E. coli*; lane 4, GST-CRAMP eluted from the column with elution buffer (100 mM glutathione); lane 5, size marker.

### Expression of the [$^{13}C/^{15}N$]-Labeled CRAMP Fusion Protein

Since the cell growth in minimal media is different from the case of LB medium, we tried to find an optimal condition again. In the case of labeled samples, the cells grew only to 70–80% of what could be expected from the LB medium. We found an optimized temperature of 30°C and 1.2 $OD_{600}$ value as the best conditions [12].

### Isolation and Purification of Recombinant CRAMP

The GST-CRAMP fusion protein was purified by a glutathione-agarose column and dialyzed against 10 mM Tris-HCl buffer, pH 8.0. The CRAMP was cleaved from the fusion protein by thrombin and purified by using reverse-phase chromatography. Purified peptide showed a peak corresponding to 4,022.2 Da, in good agreement with the theoretical value of 4,022.79. The yield of CRAMP was about 1.5 mg/l in LB or minimal media [13, 14].

### Circular Dichroism

To investigate the secondary structures of CRAMP in membrane-like environments, we measured CD spectra of CRAMP dissolved in an aqueous solution, 1:1 (v/v) 2,2,2-trifluoroethanol:$H_2O$, and 100 mM SDS micelles (Fig. 3). The CD spectra of CRAMP showed that CRAMP has a

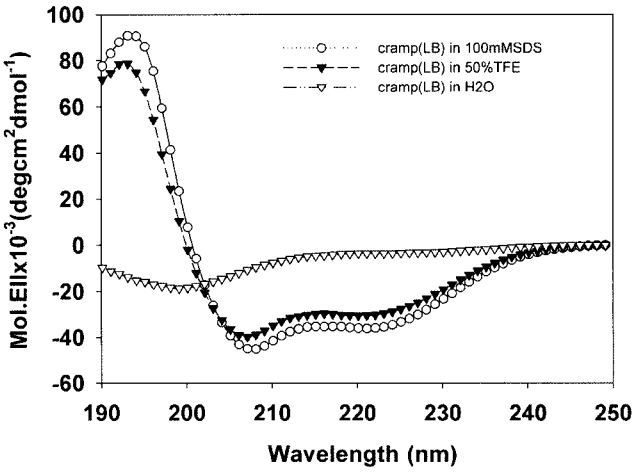


**Fig. 3.** Circular dichroism spectra of CRAMP in aqueous solution, 50% TFE/$H_2O$ solution, and 100 mM SDS micelles.

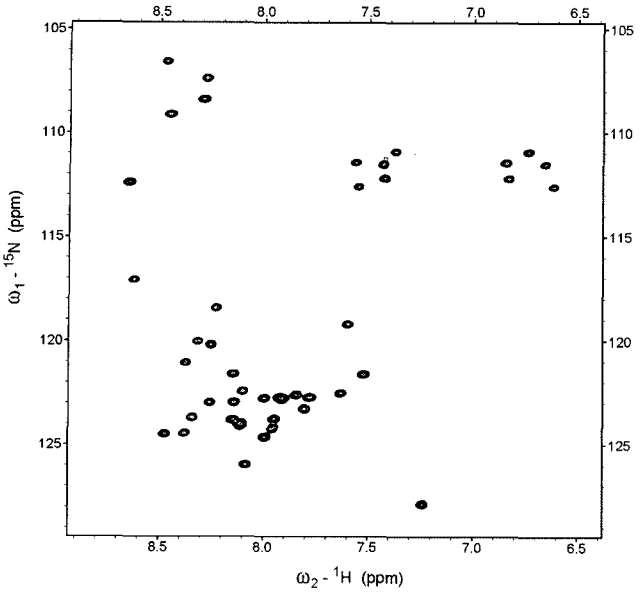


**Fig. 4.** [$^1$H-$^{15}$N] HSQC spectrum of CRAMP in 100 mM SDS micelles at 25°C.

random coil structure in aqueous solution, whereas it adopted α-helical conformations in membrane mimetic environments.

### NMR Experiments

As shown in Fig. 4, the HSQC spectrum confirmed the high purity of $^{15}$N-labeled CRAMP by showing 34 nicely resolved peaks, which correspond to the number of the residues in CRAMP, except Proline and the Gly at the N-terminus. CRAMP has six Gly residues, and they appeared between 105 ppm to 112 ppm of $^{15}$N dimensions, except for one at the N-terminus. There were five sets of peaks from the side chain of four Gln and one Asn. Although the $^1$H NMR spectrum of CRAMP in SDS micelles was difficult to analyze because of the severe overlapping, the amide resonances were dispersed in the $^1$H-$^{15}$N HSQC spectra [15].

We tried to express the antimicrobial peptide CRAMP in various fusion systems such as Ubi-fusion, KSI-fusion, and GST-fusion. Ubiquitin and KSI had problems overexpressing CRAMP, and the GST-fusion system was superior to other two systems. We optimized the overexpression and purification conditions such as temperature, pH, agitation velocity (rpm), and buffer conditions. In this study, CRAMP was successfully expressed and the resulting expression level of fusion proteins reached up to 10% of total cell proteins. Consequently, we were able to obtain the recombinant CRAMP with high purity and 1.5 mg/lyield. According to the CD spectra, CRAMP adopted an α-helical conformation in membrane-mimetic environments. In order to overcome the severe spectral overlapping problems of CRAMP in micelles, isotope labeling ($^{15}$N, $^{15}$N/$^{13}$C) was also successfully performed for the studies of the structure and dynamics of the CRAMP in micelles [15]. In conclusion, the expression system for the antimicrobial peptides described in this paper could be applied to high-level expression of various kinds of antimicrobial peptides. We are in a process to study the structure and dynamics of isotope-labeled CRAMP in various membranous environments [17].

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